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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Loss of responsiveness to TGF-β-induced cell cycle inhibition is a hallmark of cancers and the underlying mechanisms are not well understood. We now show PKB activation contributes to resistance to antiproliferative signals including TGF-β and breast cancer progression in part by impairing nuclear import and action of p27. We observed in TGF-β resistant human mammary epithelial cells, PKB is overactivated and p27 is mislocalized in the cytoplasm and the nuclear localization of p27 can be restored by PI3K inhibition. PKB transfection caused cytoplasmic p27 accumulation and resistance to cytokine-mediated G1 arrest. The nuclear localization signal of p27 contains a PKB/Akt consensus site at threonine 157 and p27 phosphorylation by PKB impaired its nuclear import *in vitro*. PKB/Akt phosphorylated wild type p27 but not p27T157A. In PKB^{DD} transfected cells, p27WT mislocalized to the cytoplasm, but p27T157A was nuclear. Taken together, our results suggested that PKB phosphorylates p27 at T157, causes cytoplasmic mislocalization and loss of Cdk inihibitory function of p27, leading to TGF-β resistance.

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INTRODUCTION

Cell cycle deregulation is a hallmark of cancer. Cytokine-mediated G1 arrest is a mechanism that controls normal cell proliferation, the loss of which may confer an advantage during malignant progression. Resistance to the antiproliferative effects of the transforming growth factor beta (TGF-β) often occurs despite intact TGF-β signaling and such cells often manifest resistance to multiple inhibitory cytokines, suggesting underlying alterations in cell cycle controls (for reviews see[1,2]).

Cell division cycle is driven by cyclin dependent kinases (cdks) that are regulated by cyclin binding, phosphorylation and by two families of cdk inhibitors[3]. G1 to S phase progression is governed by D-type and E-type cyclin-cdks. The INK4 (inhibitors of cdk4) family includes $p15^{INK4B}$ and the KIP family (kinase inhibitor protein) comprises $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (ref. 4). In addition to inhibition of cyclin E-cdk2, p21 and p27 also facilitate assembly and activation of cyclin D-cdks in early G1[5,6].

$p27^{Kip1}$ was first identified as a mediator of TGF-β1 induced G1 arrest[7-9]. TGF-β induces $p15^{INK4B}$, p27 and cyclin D1 dissociate from cyclin D1-cdk4-p27 complexes and p27 binds and inhibits cyclin E-cdk2, leading to G1 arrest[10-12]. p27 is a nuclear protein whose frequent deregulation in human cancers may confer resistance to antiproliferative signals, such as TGF-β.

In human cancers, constitutive PI3K/PKB activation arises through oncogenic receptor tyrosine kinase activation, *Ras* activation, mutational loss of *PTEN*, or through activating mutation of the PI3K effector, *PKB/Akt* itself[21,22]. PKB/Akt (hereafter referred to as PKB) can increase cyclin D1 levels[23] and down-regulate p27 by increasing p27 proteolysis[24] or repressing *p27* expression through PKB phosphorylation of a forkhead

transcription factor[25]. However, in most cancers, reduced p27 does not result from transcriptional silencing[19]. This grant proposed to explore the possibility that **mitogenic pathways acting via phosphoinositol 3' kinase (PI3K) phosphorylate p27, reduce its affinity for cyclin E/cdk2 and abrogate its inhibitory function and that constitutive activation of the PI3K/PKB pathway can cause TGF-β resistance in human mammary epithelial cells (HMEC).**

This hypothesis is currently being pursued by the following specific aims. AIM 1) Functional differences in p27 will be examined during cell cycle progression in TGF-β S and R lines by: i) assaying differences in p27's affinity for and inhibition of recombinant cyclin E/cdk2 and ii) correlating changes in p27 function with shifts in its intracellular localization. AIM 2) The role of phosphorylation in modulation of p27 function will be assayed by 2DIEF of p27 in different cyclin/cdk complexes from S and R lines. In AIM 3), effects of constitutive activation of the PI3K/PKB signal transduction pathways on p27 activity and TGF-β sensitivity will be assayed by transforming HMEC with activated PI3K or PKB. This report will summarize the progress of these investigations.

PROGRESS SUMMARY

1. Increased activation of PKB in lines resistant to G1 arrest by TGF-β

To address the question whether PI3K/PKB plays a role in TGF-β resistance, we first examined the correlation between PKB activation and TGF-β resistance. We found TGF-β causes G1 arrest of normal 184 HMEC but not the resistant 184A1L5^{R} (Fig. 1*a*) that showed increased PKB activation (PKB-P) (Fig. 1*b*). Total PKB was similar in sensitive and resistant HMEC. The increased PI3K/PKB activity in 184A1L5^{R} was linked to their TGF-β resistance, since partial PI3K inhibition restored TGF-β responsiveness. In 184A1L5^{R}, low concentrations of the PI3K inhibitor, LY294002 (10-12.5 μM) modestly reduced the % S phase fraction but allowed continued proliferation, with a profile similar to that of 184 cells (Fig. 1*c*). Although TGF-β alone had little effect, 10 μM LY294002 together with TGF-β caused G1 arrest of 184A1L5^{R} (Fig. 1*c*).

Since inhibition of cyclin E-cdk2 by p27 contributes to G1 arrest by TGF-β, cyclin E1 complexes were assayed in 184AIL5^{R}. Low dose LY294002 (10 μM) inhibited PKB activity (Fig. 1*d*). LY294002 did not change p27 protein levels in the HMEC, but modestly increased cyclin E1 bound p27 and partly inhibited cyclin E1-cdk2 (Fig. 1*d*). TGF-β and LY294002 together increased further cyclin E1 bound p27 and inhibited cyclin E1-cdk2 causing G1 arrest. Thus, attenuation of P13K activity restored cyclin E1-cdk2 inhibition by p27 and G1 arrest by TGF-β.

2. Constitutive PKB activation inhibits responsiveness to TGF-β

To further ascertain whether PKB activation is sufficient to cause TGF-β resistance, 184 cells were transfected with a constitutively active PKB^{T308DS473D} (PKB^{DD})[26,27] or empty

vector(E). Transfectants showed increased PKB protein and activity (Fig. 2). PKB^{DD} transfected lines were partially resistant to G1 arrest by TGF-β.

3. A PKB-dependent pathway causes cytoplasmic mislocalization of p27 in TGF-β resistant cells

Cellular p27 was predominantly nuclear in 184. $184A1L5^{R}$ and 184^{PKBDD} cells showed both nuclear and cytoplasmic p27 (Fig. 3). Transfected fluorescent-tagged wild type p27 (YFPp27WT) was exclusively nuclear in most 184. YFPp27WT transfected $184A1L5^{R}$ cells showed increased cytoplasmic p27. LY294002 restored the predominantly nuclear localization of YFPp27WT in TGF-β resistant $184A1L5^{R}$. In 184^{PKBDD} transfected YFPp27WT showed increased cytoplasmic localization, similar to that in $184A1L5^{R}$.

4. PKB/Akt binds and phosphorylates cellular $p27^{Kip1}$

A minimal consensus motif has been defined for PKB[29]. p27 contains a putative PKB consensus sequence between amino acids 152 to 157. Immunoprecipitated cellular PKB could phosphorylate recombinant p27 directly *in vitro* as well as the known PKB substrate, histone H2B. p27T157A, generated by replacing the T157 with alanine, was much less efficiently phosphorylated by PKB (Fig. 4*a*). Thus, T157 is identified as a major PKB phosphorylation site in p27. The inability of PKB to phosphorylate p27T157A is not due to loss of PKB binding since both endogenous p27 and ectopically expressed p27WT and p27T157A co-precipitated with activated cellular PKB-P (Fig. 4*b*).

To provide evidence for *in vivo* phosphorylation of p27 by PKB, cellular p27 immunoprecipitates from asynchronous and LY294002 treated MCF-7 cells were immunoblotted with a phospho-specific antibody against the PKB/Akt phosphorylation consensus motif. Immunoreactivity with this antibody demonstrated cellular p27

phosphorylation at the PKB consensus motif in asynchronous cells. This was inhibited by LY294002 (Fig. 4*c*).

KEY RESEARCH ACCOMPLISHMENTS

- PKB is over-activated in the TGF-β resistant HMEC
- Inhibition of the PI3K/PKB pathway restored sensitivity to TGF-β in resistant lines
- Expression of constitutively active PKB led to TGF-β resistance
- P27 is mislocalized in cytoplasm in TGF-β resistant lines
- Nuclear localization of p27 can be restored by blocking the PI3K/PKB pathway
- PKB phosphorylates p27 at threonine 157
- Mutation of threonine 157 to alanine abolished PKB-dependent cytoplasmic mislocalization of p27

REPORTABLE OUTCOMES

1. Jiyong Liang, Judit Zubovitz, Teresa Petrocelli, Rouslan Kotchetkov, Michael K. Connor, Kathy Han, Jin-Hwa Lee, Sandra Ciarallo, Charles Catzavelos, Richard Beniston, Edmee Franssen and Joyce M. Slingerland. PKB phosphorylates p27, impairs its nuclear import and opposes p27-mediated G1 arrest. Nature Medicine, 8(10): 1153-1160, 2002.
2. Jiyong Liang, Judit Zubovitz, Teresa Petrocelli, Rouslan Kotchetkov, Michael K. Connor, Richard Beniston, Kathy Han, Jin-Hwa Lee, Sandra Ciarallo, Charles Catzavelos, Edmee Franssen and Joyce M. Slingerland. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and mediates resistance to TGF-beta-mediated G1 arrest in human cancers. Poster presentation, Department of Defense Breast Cancer Research Program Meeting, September, 2002, Orlando, FL
3. Jiyong Liang, Kathy Han, Wesley Hung, Joyce M. Slingerland. Akt/PKB-dependent phosphorylation of p27 activates the cyclin D1/Cdk4 assembly function of p27 and G1 cell cycle progression. Platform presentation, The Cell Cycle Meeting, Cold Spring Harbor, 2002.

CONCLUSIONS

We have shown that in TGF-β resistant cells, the PI3K/PKB pathway is over-activated and p27 is mislocalized in the cytoplasm. We also demonstrated that PKB phosphorylates p27 at threonine157 within the nuclear localization sequence of p27 and that inhibition of the PI3K/PKB pathway restored nuclear localization of p27 and the sensitivity to TGF-β induced G1 arrest in TGF-β resistant cells. These findings suggest a mechanistic model that PKB phosphorylates p27 and causes p27 to be sequestered in the cytoplasm, away from its nuclear target, cyclin E/cdk2, leading to resistance to TGF-β-mediated cell cycle arrest. Our findings suggest that the PI3K/PKB pathway, which is commonly over-activated in breast cancers via a number of mechanisms such as over-expression of *HER2* and *Akt1* or loss of tumor suppressor PTEN and p53, is a potential target for cancer therapeutic intervention.

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APPENDICES

1. Figure 1 to Figure 4
2. Manuscript submitted: PKB phosphorylates p27, impairs its nuclear import and opposes p27-mediated G1 arrest.

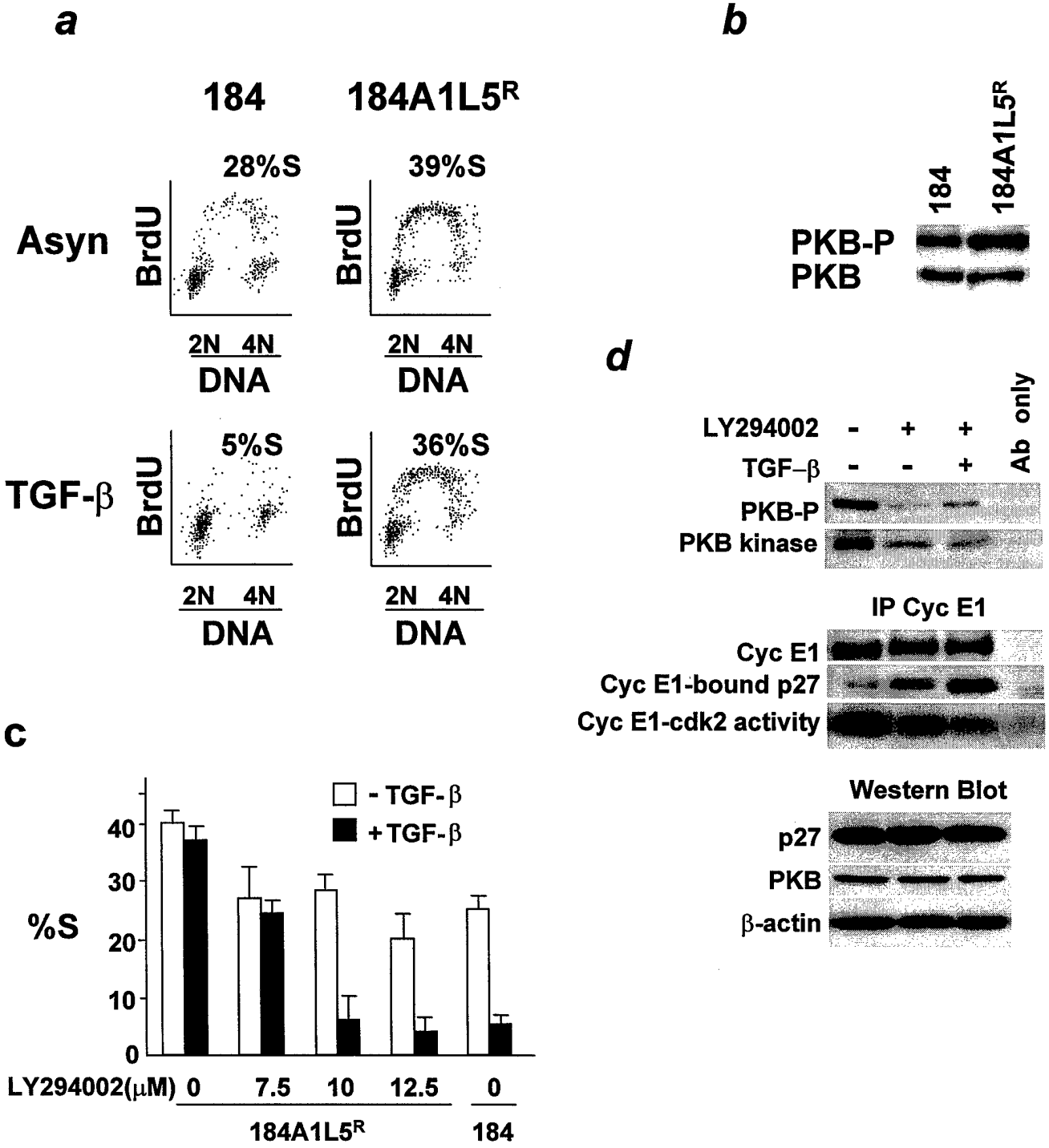
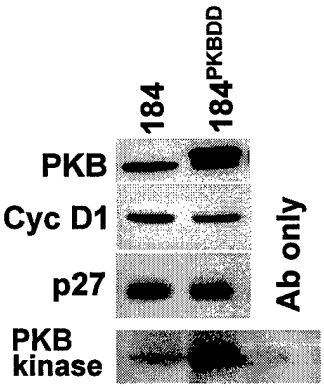


Fig. 1. Constitutive activation of PKB/Akt in TGF-β resistant cell lines. Proliferating cells were treated without (Asyn) or with TGF-β for 48 hrs. ***a*** shows flow cytometric analysis with or without TGF-β. ***b*** shows immunoblots of activated PKB (PKB-P) and total PKB. ***C*** and ***d*** Cells were treated with LY294002 alone or with TGF-β for 24 hrs. ***c***.Effects on the % S phase ***d***, Effects in 184A1L5^R of 10 μM LY294002 with or without TGF-β on PKB-P levels, PKB kinase activities, cyclin E1-association with p27 and cyclin E-cdk2 activities. Lane 4 shows the antibody only control for the top 2 panels. Total PKB, p27 and β-actin levels are shown in the bottom panel.

a



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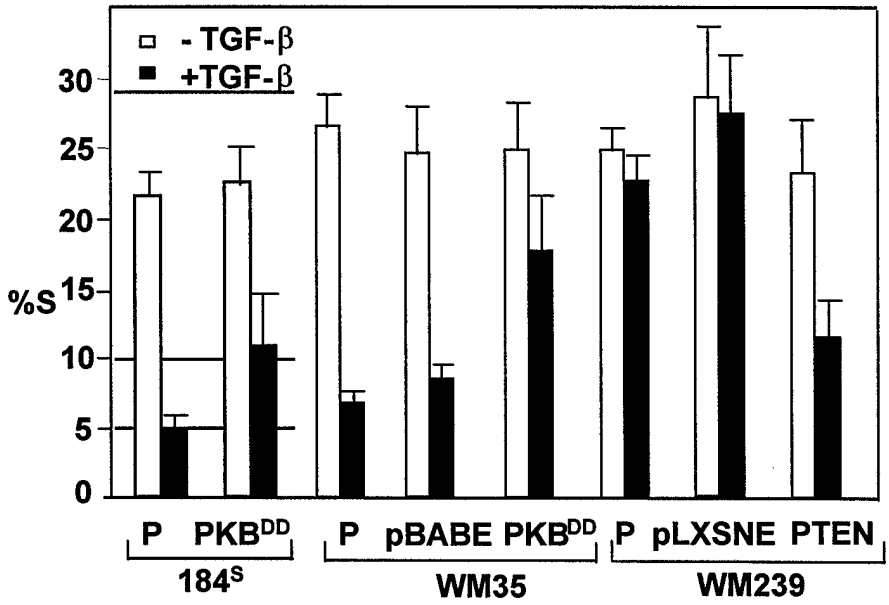

Fig. 2. PKB transfection mediates TGF-β resistance. ***a*** 184 cells were transformed with PKB^{DD}, and the ectopic expression of PKB^{DD} was shown by Western blot (PKB) and PKB activity confirmed by PKB kinase assays (PKB kinase). ***b*** Flow cytometric analysis of parental and transfected cells treated with or without TGF-β for 48 hrs.

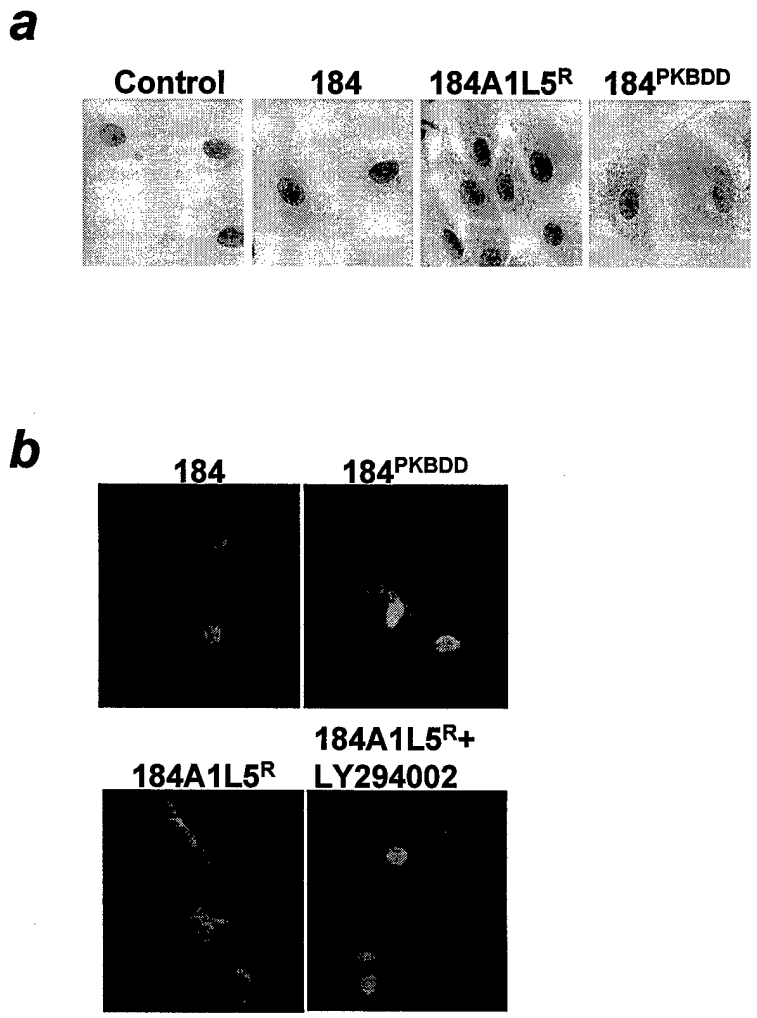

Fig. 3. Cytoplasmic mislocalization of p27 in TGF-β resistant, PKB activated cells. ***a***, Cellular p27 detected by immunocytochemistry. Cells in the control panel were stained with isotopic specific polyclonal IgG and counterstained with hematoxylin. ***b***, Cells were transfected with YFPp27WT or treated with LY294002 and observed by direct fluorescence microscopy.

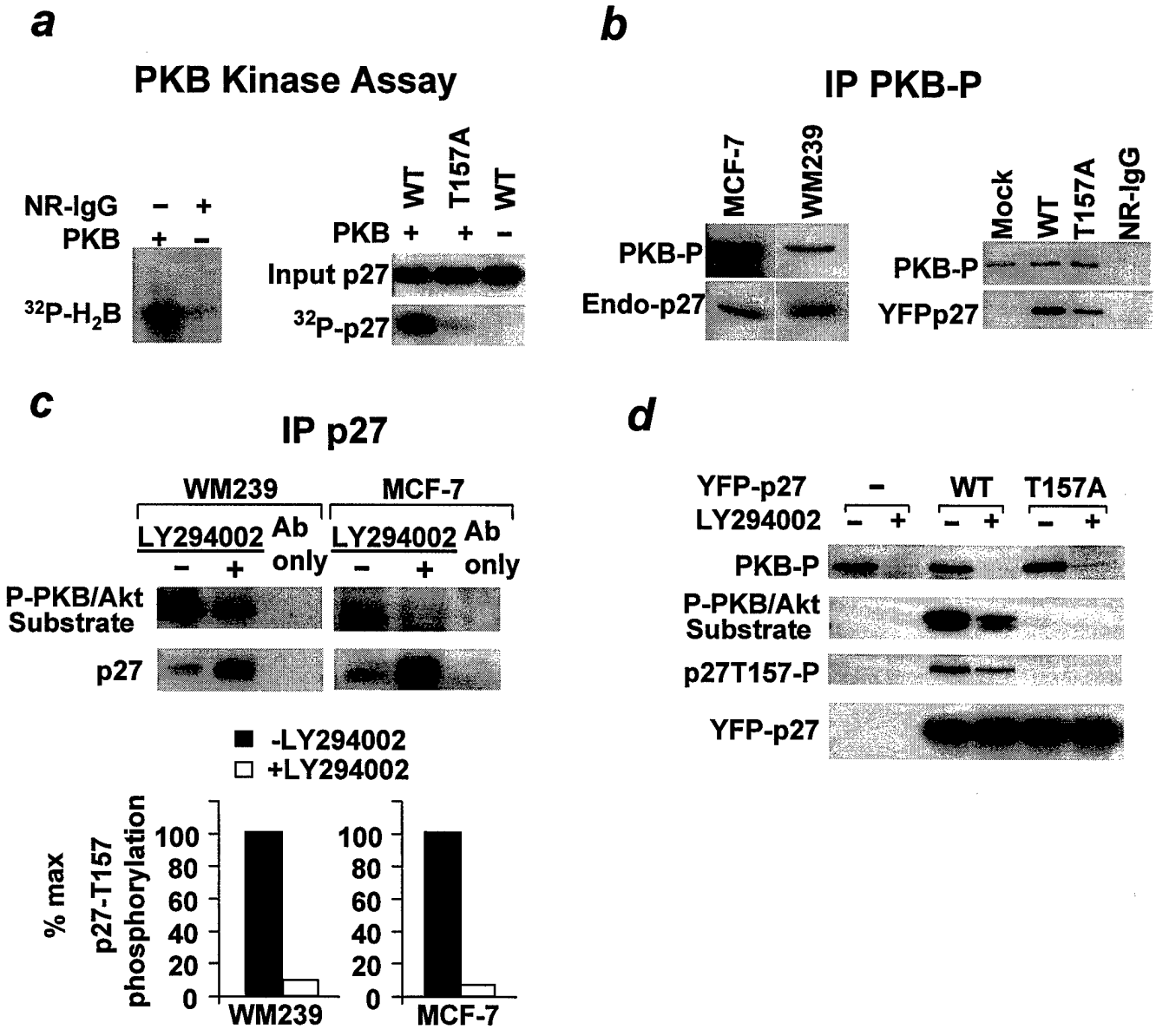

Fig. 4. p27 is substrate of PKB and binds PKB *in vivo*. ***a***, PKB kinase assays using either histone H2B, or recombinant wild-type p27 (p27WT) as substrates. Control reactions using normal rabbit IgG (NR-IgG) are shown (right lanes). PKB phosphorylated p27WT but not p27T157A in *in vitro* kinase assays. Input p27 is immunoblotted in the top right panel. ***b***, Ser473-phosphorylated PKB immunoprecipitates were resolved and immunoblotted to show PKB-P and associated endogenous cellular p27 (left panel) or transfected YFPp27WT and YFPp27T157A (right panel). ***c***, Cellular p27 shows reactivity with antibody specific for the phosphorylated PKB/Akt consensus motif. While p27 levels are increased by LY294002, reactivity with the phospho-PKB/Akt substrate antibody (P-PKB/Akt substrate) is diminished. ***d***, WM239 cells were transfected with YFPp27WT or YFPp27T157A and then treated with or without LY294002 for 20 hours. The top band shows PKB-P. YFPp27 was immunoblotted with antibodies to P-PKB/Akt substrate, anti-p27T157-P and total p27.

PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest

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Mechanisms linking mitogenic and growth inhibitory cytokine signaling and the cell cycle have not been fully elucidated in either cancer or in normal cells. Here we show that activation of protein kinase B (PKB)/Akt contributes to resistance to antiproliferative signals and breast cancer progression in part by impairing the nuclear import and action of p27. Akt transfection caused cytoplasmic p27 accumulation and resistance to cytokine-mediated G1 arrest. The nuclear localization signal of p27 contains an Akt consensus site at threonine 157, and p27 phosphorylation by Akt impaired its nuclear import *in vitro*. Akt phosphorylated wild-type p27 but not p27T157A. In cells transfected with constitutively active Akt$^{T308D S473D}$ (PKB^{DD}), p27WT mislocalized to the cytoplasm, but p27T157A was nuclear. In cells with activated Akt, p27WT failed to cause G1 arrest, while the antiproliferative effect of p27T157A was not impaired. Cytoplasmic p27 was seen in 41% (52 of 128) of primary human breast cancers in conjunction with Akt activation and was correlated with a poor patient prognosis. Thus, we show a novel mechanism whereby Akt impairs p27 function that is associated with an aggressive phenotype in human breast cancer.

Cell-cycle deregulation is a hallmark of cancer. Loss of cytokine-mediated G1 arrest may confer an advantage during malignant progression. Resistance to the antiproliferative effects of transforming growth factor-β (TGF-β) often occurs despite intact TGF-β signaling and such cells may manifest resistance to multiple inhibitory cytokines, suggesting underlying alterations in cell-cycle controls[1,2].

Cyclin-dependent kinases (cdks) are regulated by cyclin binding, phosphorylation and by two families of cdk inhibitors[3]. G1 progression is governed by D-type and E-type cyclin-cdk complexes. The inhibitors of cdk4 (INK4) family includes p15^{INK4B} and the kinase inhibitor protein (KIP) family comprises p21^{Cip1}, p27^{Kip1} and p57^{Kip2} (ref. 4). In addition to inhibition of cyclin E-cdk2, p21 and p27 also facilitate assembly and activation of cyclin D-cdks in early G1 (refs. 5,6).

p27^{Kip1} was first identified as a mediator of TGF-β1-induced G1 arrest[7–9]. TGF-β induces expression of the gene encoding *p15^{INK4B}*. p15^{INK4B} binds and inhibits cdk4 facilitating dissociation of p27 and cyclin D1 from cyclin D1–cdk4–p27 complexes and p27 binds and inhibits cyclin E-cdk2, leading to G1 arrest[10–12]. p27 also mediates G1 arrest induced by IL-6 (ref. 13). p27 is a nuclear protein whose frequent deregulation in human cancers may confer resistance to antiproliferative signals. In cMyc or MAPK activated cancer-derived lines, cyclin D1–cdk4/6 complexes sequester p27, and cyclin E-cdk2-inhibition is impaired[14–16]. cMyc inhibits p15 induction by TGF-β (ref. 17) and may also induce a factor that inactivates p27[18]. In up to 50% of human cancers, reduced p27 protein is associated with a poor prognosis[19]. In some tumors, p27 is mislocalized to the cytoplasm[19,20], however; the mechanism and significance of this has not been elucidated.

In human cancers, constitutive activation of phosphoinositol 3′ kinase (PI3K) and its effector PKB/Akt arise through oncogenic receptor tyrosine kinase activation, *Ras* activation, mutational loss of *PTEN*, or through activating mutation of the PI3K effector, protein kinase B (PKB)/Akt (hereafter termed Akt) itself[21,22]. Akt can increase cyclin D1 levels[23] and downregulate p27 by increasing p27 proteolysis[24] or repressing p27 expression through Akt phosphorylation of a forkhead transcription factor[25]. However, in most cancers, reduced p27 does not result from transcriptional silencing[19].

Here we show that Akt causes resistance to cytokine-mediated G1 arrest. p27 phosphorylation by Akt impairs its nuclear import and leads to cytoplasmic p27 accumulation. In human breast cancers, cytoplasmic mislocalization of p27 is associated with Akt activation, loss of differentiation and poor patient outcome.

Activation of Akt in lines resistant to G1 arrest by TGF-β

Ras has been shown to confer TGF-β resistance. While investigating mechanisms of TGF-β resistance, we found that two TGF-β

resistant lines showed activation of the Ras effector, PI3K-Akt pathway. TGF-β causes G1 arrest of normal 184 human mammary epithelial cells (HMECs) and of the early stage melanoma line, WM35, but not the resistant 184A1L5^R or advanced melanoma-derived line, WM239 (Fig. 1*a* and *b*). Resistant lines showed increased Akt activation (Akt-P) (Fig. 1*c* and *d*). Total Akt was similar in sensitive and resistant HMECs. Akt levels were somewhat higher in WM239 than WM35. PTEN loss contributed to Akt activation in WM239 (Fig. 1*d*).

The increased PI3K-Akt activity in 184A1L5^R and WM239 was linked to their TGF-β resistance, as partial PI3K inhibition restored TGF-β responsiveness. In 184A1L5^R, low concentrations of the PI3K inhibitor, LY294002 (10–12.5 μM) modestly reduced the S phase fraction (% S) but allowed continued proliferation, with a profile similar to that of 184 cells (Fig. 2*a*). Although TGF-β alone had little effect, 10 μM LY294002 together with TGF-β caused G1 arrest of 184A1L5^R (Fig. 2*a*). In TGF-β-resistant WM239, LY294002 at a concentration that did not independently inhibit proliferation led to G1 arrest when combined with TGF-β (data not shown).

Because inhibition of cyclin E-cdk2 by p27 contributes to G1 arrest by TGF-β, cyclin E1 complexes were assayed in 184AIL5^R. A low dose of LY294002 (10 μM) inhibited Akt activity (Fig. 2*b*). LY294002 did not change p27 protein levels in HMECs (Fig. 2*b*), but modestly increased cyclin E1–bound p27 and partly inhibited cyclin E1-cdk2 activity (Fig. 2*b*). TGF-β and LY294002 together increased further cyclin E1–bound p27 and inhibited cyclin E1-cdk2 causing G1 arrest. Thus, attenuation of P13K activity restored cyclin E1-cdk2 inhibition by p27 and G1 arrest by TGF-β.

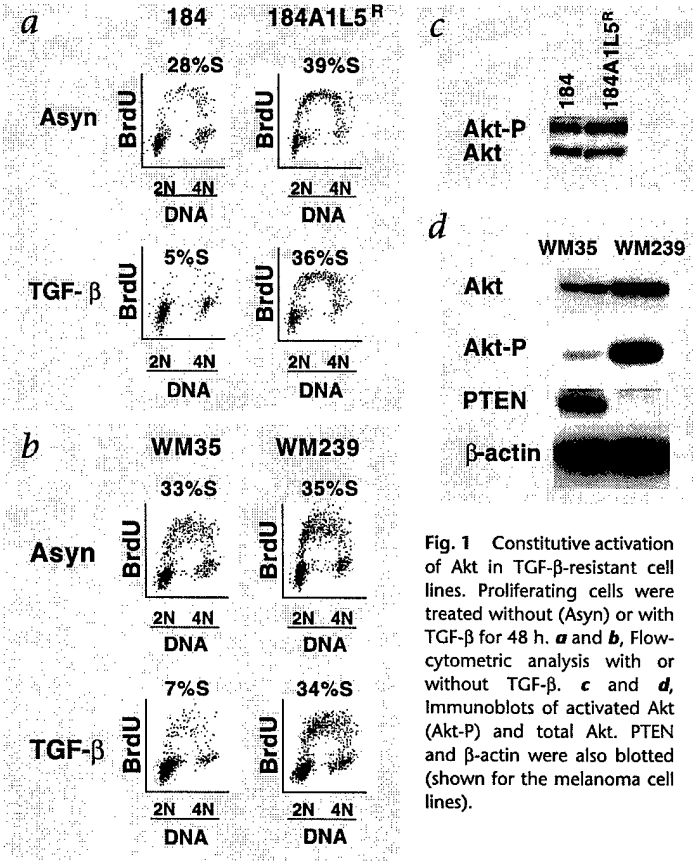

Fig. 1 Constitutive activation of Akt in TGF-β-resistant cell lines. Proliferating cells were treated without (Asyn) or with TGF-β for 48 h. ***a*** and ***b***, Flow-cytometric analysis with or without TGF-β. ***c*** and ***d***, Immunoblots of activated Akt (Akt-P) and total Akt. PTEN and β-actin were also blotted (shown for the melanoma cell lines).

Akt inhibits responsiveness to antiproliferative cytokines

184 and WM35 cells were transfected with a constitutively active Akt^{T308DS473D} (PKB^{DD})[26,27] or empty vector (E). Transfectants showed increased Akt protein and activity (Fig. 2*c*). PKB^{DD}-transfected lines were resistant to G1 arrest by TGF-β (shown for WM35^{PKBDD})

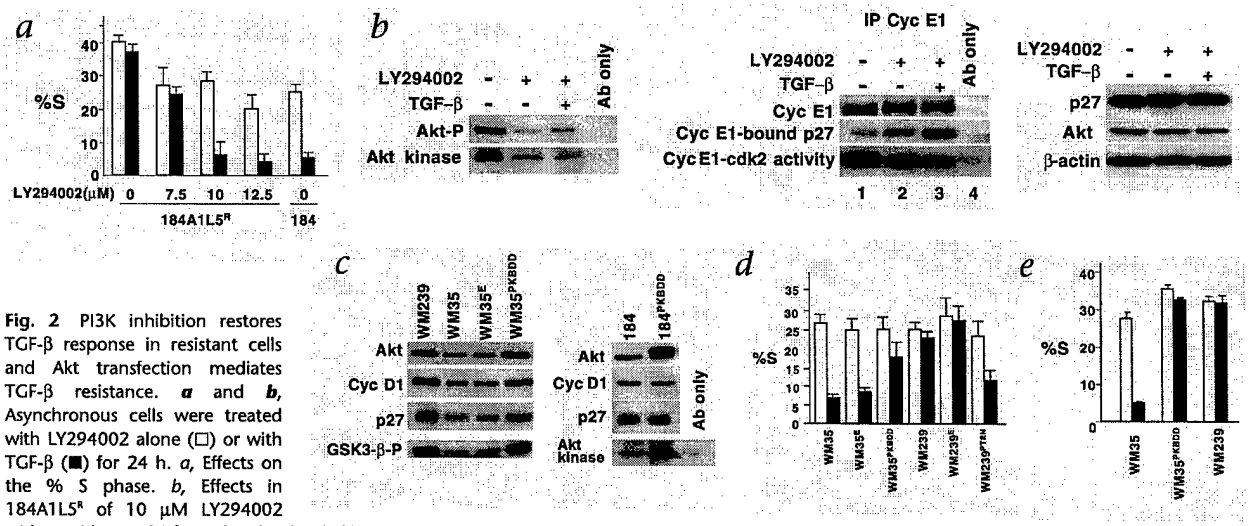

Fig. 2 PI3K inhibition restores TGF-β response in resistant cells and Akt transfection mediates TGF-β resistance. ***a*** and ***b***, Asynchronous cells were treated with LY294002 alone (□) or with TGF-β (■) for 24 h. *a*, Effects on the % S phase. *b*, Effects in 184A1L5^R of 10 μM LY294002 with or without TGF-β on Akt-P levels, Akt kinase activities, cyclin E1-association with p27 and cyclin E-cdk2 activities. Lane 4 shows the antibody only control for the left and middle panels. Total Akt, p27 and β-actin levels are shown in the right panel. ***c–e***, The indicated cells were transformed with PKB^{DD}, PTEN or empty vector (E). *c*, Ectopic expression of PKB^{DD} was shown by western blot (Akt) and Akt activity confirmed by blotting for phosphorylation of the Akt substrate, GSK3-β (GSK3-β-P) or by Akt kinase assays (Akt kinase). Levels of p27 and cyclin D1 are shown. *d* and *e*, Flow cytometric analysis of parental and transfected cells treated with (■) or without (□) TGF-β (*d*) and without (□) or with (■) IL-6 (*e*) for 48 h.

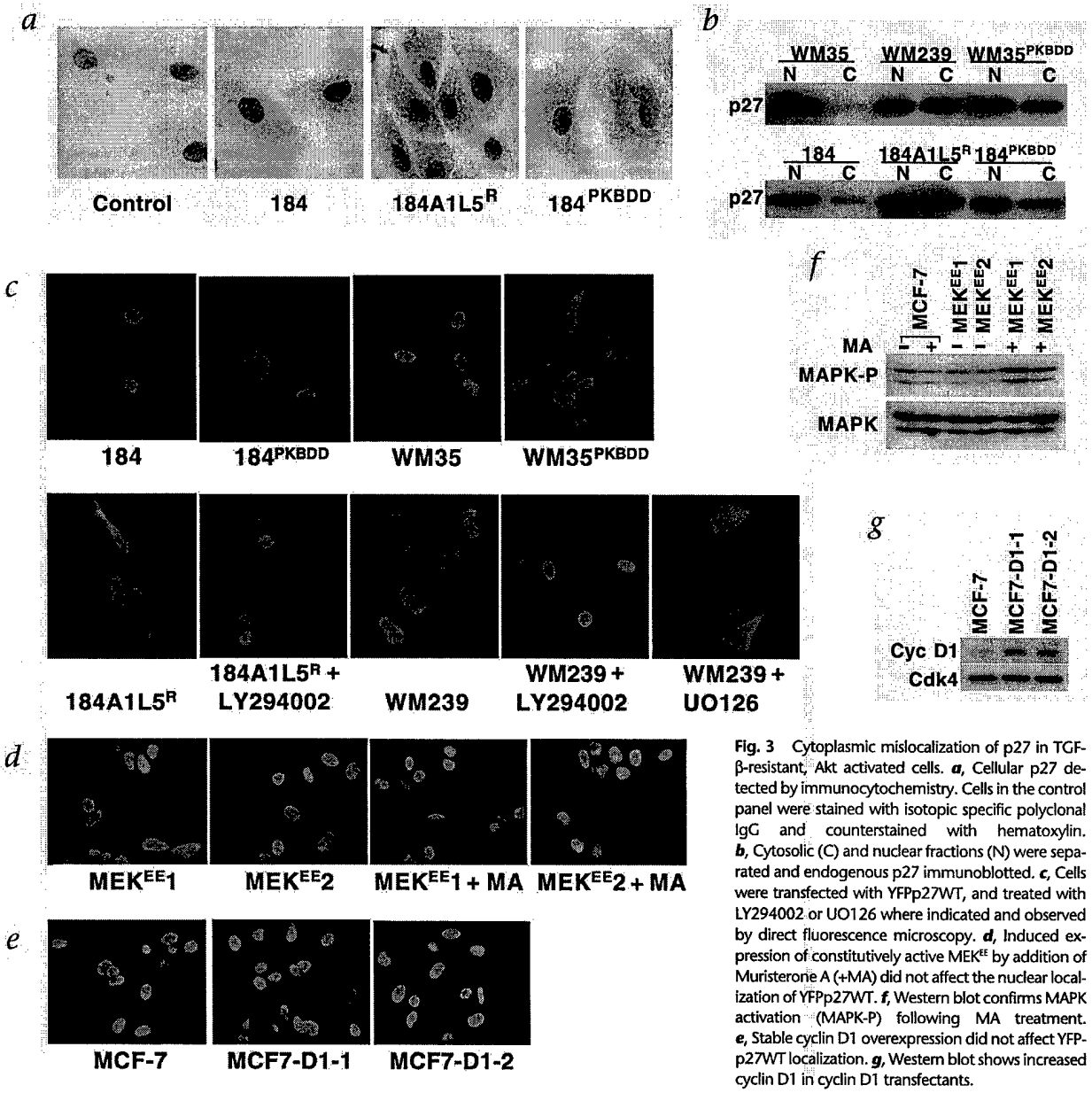

Fig. 3 Cytoplasmic mislocalization of p27 in TGF-β-resistant, Akt activated cells. ***a***, Cellular p27 detected by immunocytochemistry. Cells in the control panel were stained with isotopic specific polyclonal IgG and counterstained with hematoxylin. ***b***, Cytosolic (C) and nuclear fractions (N) were separated and endogenous p27 immunoblotted. ***c***, Cells were transfected with YFPp27WT, and treated with LY294002 or UO126 where indicated and observed by direct fluorescence microscopy. ***d***, Induced expression of constitutively active MEK^{EE} by addition of Muristerone A (+MA) did not affect the nuclear localization of YFPp27WT. ***f***, Western blot confirms MAPK activation (MAPK-P) following MA treatment. ***e***, Stable cyclin D1 overexpression did not affect YFP-p27WT localization. ***g***, Western blot shows increased cyclin D1 in cyclin D1 transfectants.

(Fig. 2*d*). Moreover, PKB^{DD} transfection also conferred resistance to G1 arrest by IL-6 (Fig. 2*e*). In WM239, transfection of PTEN restored TGF-β sensitivity (Fig. 2*d*).

Akt causes cytoplasmic mislocalization of p27

Because Akt impairs the nuclear localization of some of its substrates[28], we assayed if Akt activation affected p27 localization. Cellular p27 was predominantly nuclear in 184 HMEC. $184A1L5^R$ and 184^{PKBDD} cells showed both nuclear and cytoplasmic p27 (Fig. 3*a*). Increased cytoplasmic p27 in WM239 and $WM35^{PKBDD}$, and in $184A1L5^R$ and 184^{PKBDD} compared with WM35 and 184, respectively, was confirmed by immunoblotting of fractionated cell lysates (Fig. 3*b*).

Transfected fluorescent-tagged wild-type p27 (YFPp27WT) was exclusively nuclear in most 184 and WM35 cells (Fig. 3*c* and Supplementary Table A online). YFPp27WT-transfected $184A1L5^R$ and WM239 showed increased cytoplasmic p27. LY294002 restored the predominantly nuclear localization of YFPp27WT in TGF-β-resistant $184A1L5^R$ and WM239 cells (Fig. 3*c*). In 184^{PKBDD} and $WM35^{PKBDD}$, transfected YFPp27WT showed increased cytoplasmic localization, similar to that in $184A1L5^R$ and WM239.

In contrast to effects of LY294002, treatment of WM239 cells with the MEK inhibitor, UO126, did not result in redistribution of p27 from cytoplasm to nucleus. Moreover, in two independent cell lines, inducible overexpression of constitutively activated MEK (MEK^{EE}) and MAPK activation did not cause

cytoplasmic mislocalization of p27 (Fig. 3*d* and *f*). Thus, in this culture model, MAPK activation is neither necessary nor sufficient for cytoplasmic mislocalization of p27. As both Akt and MAPK activation can increase cyclin D1 levels, we tested the effect of cyclin D1 transfection on p27 localization. Cyclin D1 overexpression did not mediate cytoplasmic localization of p27 (Fig. 3*e* and *g*).

Akt binds and phosphorylates cellular p27^{Kip1}

A minimal consensus motif has been defined for Akt (ref. 29). p27 contains a putative Akt consensus sequence between amino acids 152 and 157 (RKRPAT). Immunoprecipitated cellular Akt could phosphorylate recombinant p27 directly *in vitro* as well as the known Akt substrate, histone H2B. p27T157A, generated by replacing the T157 with alanine, was much less efficiently phosphorylated by Akt (Fig. 4*a*). Thus, T157 is identified as a putative Akt phosphorylation site in p27. The inability of Akt to phosphorylate p27T157A is not due to loss of Akt binding as both endogenous p27 and ectopically expressed p27WT and p27T157A coprecipitated with activated cellular Akt-P (Fig. 4*b*).

To demonstrate that phosphorylation of p27 by Akt can occur in cells, p27 immunoprecipitates from asynchronous and LY294002-treated WM239 and MCF-7 cells were immunoblotted with a phospho-specific antibody against the Akt phosphorylation consensus motif (P-Akt substrate antibody). Immunoreactivity with this antibody demonstrated cellular p27 phosphorylation at the Akt consensus motif in asynchronous cells. This was inhibited by LY294002 (Fig. 4*c*).

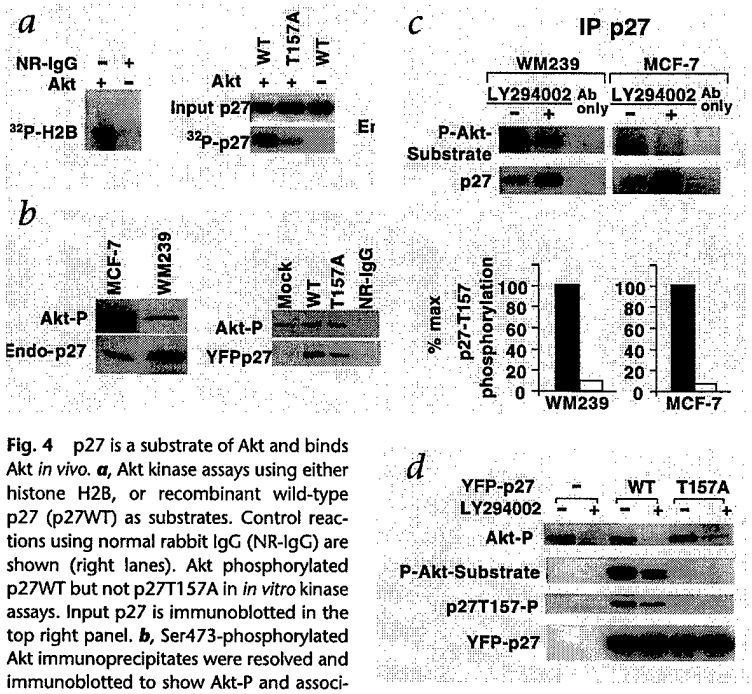

Fig. 4 p27 is a substrate of Akt and binds Akt *in vivo*. ***a***, Akt kinase assays using either histone H2B, or recombinant wild-type p27 (p27WT) as substrates. Control reactions using normal rabbit IgG (NR-IgG) are shown (right lanes). Akt phosphorylated p27WT but not p27T157A in *in vitro* kinase assays. Input p27 is immunoblotted in the top right panel. ***b***, Ser473-phosphorylated Akt immunoprecipitates were resolved and immunoblotted to show Akt-P and associated endogenous cellular p27 (left panel) or transfected YFPp27WT and YFPp27T157A (right panel). ***c***, Cellular p27 shows reactivity with antibody specific for the phosphorylated Akt consensus motif. While p27 levels are increased by LY294002, reactivity with the phospho-Akt substrate antibody (P-Akt substrate) is diminished. Lower graph: ■, without LY294002; □, with LY294002. ***d***, WM239 cells were transfected with YFPp27WT or YFPp27T157A and then treated with or without LY294002 for 20 hours. The top band shows Akt-P. YFPp27 was immunoblotted with antibodies to P-Akt substrate, anti-p27T157-P and total p27.

In normal 184 HMECs, Akt-P is minimal in G0 and increases rapidly when cells enter early G1 (data not shown). Using an antibody specific for T157-phosphorylated p27 generated by Viglietto *et al.*, we showed that cellular T157 phosphorylated p27 was minimal in G0 and the relative amount of T157 phosphorylated p27 over total p27 rose by 1.8-fold within 4 hours of exit from quiescence (data not shown). Densitometric analysis of p27T157-P and total p27 blots showed that the relative amount of T157 phosphorylated over total p27 was two-fold higher in proliferating WM239 than in WM35 (data not shown). Thus, activation of Akt *in vivo* is associated with increased T157 phosphorylation of p27 in both normal HMECs and in tumor-derived cells.

To provide further evidence for p27 phosphorylation by Akt *in vivo*, WM239 cells were transfected with either YFPp27WT or YFPp27T157A and treated with 20 μM LY294002. LY294002 markedly reduced reactivity of YFPp27WT with the antibody to the phosphorylated Akt consensus motif (P-Akt substrate) and the p27T157-P antibody. p27T157A showed minimal reactivity with the antibody to phosphorylated Akt substrate and none to anti-p27T157-P (Fig. 4*d*).

p27T157A is nuclear in cells with constitutive Akt

T157 is located within the nuclear localization signal (NLS) of p27[30,31]. Phosphorylation of the NLS regulates nuclear localization of many proteins[32]. To assay whether the potential for T157 phosphorylation might influence p27 localization, WM35^{PKBDD} cells were transfected with YFPp27WT, YFPp27T157A and YFPp27T157D (Fig. 5*a* and Supplementary Table B online). While approximately 30% of YFPp27WT and YFPp27T157D expressing cells showed both nuclear and cytoplasmic p27, YFPp27T157A was nuclear.

WM239 cells, whose Akt is constitutively activated, were transiently transfected with either YFPp27WT or YFPp27T157A. Flow cytometric analysis of YFP positive cells at 20 hours post-transfection revealed that the cell-cycle inhibitory function of p27WT was significantly impaired, while p27T157A retained G1 inhibitory function in WM239 (Fig. 5*b*). Equal expression of YFPp27WT and YFPp27T157A was demonstrated (Fig. 4*d*, lower). In contrast, YFPp27WT and YFPp27T157A both caused G1 arrest in 184 HMECs and WM35 cells lacking constitutive Akt activation (data not shown). Thus p27 phosphorylation by Akt impairs its G1 inhibitory function.

Phosphorylation by Akt impairs nuclear import of p27

p27 nuclear import was assessed by the incubation of His-tagged p27 (His-p27) with digitonin permeabilized MCF-7 cells. Nuclei and supernatant fractions were then immunoblotted for His-p27. Prior reaction of recombinant His-p27WT with cellular Akt kinase impaired nuclear import of p27. His-p27T157A showed a faster rate of import than His-p27WT, and His-p27T157D protein showed essentially no nuclear import above that of negative controls (reactions carried out at 4 °C, without ATP or in the presence of wheat germ agglutinin) (Fig. 5*c*). These data suggest that cytoplasmic p27 in Akt activated cells results from impaired p27 nuclear import.

Cytoplasmic p27 and activated Akt in human breast cancers

p27 protein levels and localization were reviewed in 128 primary breast cancers previously stained for p27 by immunohistochemistry[33]. Levels of p27 were scored as high (>50%) or low (≤50%) tumor nuclei staining as previously described[33]. While normal breast ductal epithelium and lymphocytes and a majority of tumors (Fig. 6*a*) showed exclusively nuclear p27, 42% (52/128) of the breast cancers showed either nuclear and cytoplasmic or predominantly cytoplasmic p27 (Fig. 6*b* and *c*). Of cancers with reduced p27 levels, 44% (31/70) showed cytoplasmic p27, while 36% (21/58) of tumors with high p27 levels showed cytoplasmic p27. p27 protein levels and cytoplasmic p27 mislocalization were not statistically correlated (chi square analysis, $P = 0.3542$).

Cytoplasmic p27 was not correlated with menopausal, nodal or ER/PR status. Tumors with high levels of exclusively nuclear p27 (N only; p27 >50% nuclei positive) (Fig. 6*a*) were almost all well differentiated or of low grade, whereas tumors with high levels of p27 but cytoplasmic localization (N+C; p27 >50%) (Fig. 6*b*) were more poorly differentiated ($P < 0.001$ on χ^2 analysis) (Supplementary Table C). The Kaplan–Meier curve in Fig. 6*d* shows the influence of p27 localization on patient survival. Fig. 6*e* shows that for each level of p27 staining (high, >50% nuclei positive or low, ≤50% nuclei positive), when p27 is seen in the cytoplasm (N+C) rather than in the nucleus alone (N), patient survival is worse. These data were statistically significant for overall survival ($P = 0.05$, Wilcoxon test) (Fig. 6*e*) and for disease-free survival ($P = 0.003$, data not shown). Patients with breast cancers with high levels of exclusively nuclear p27 (N only; p27 >50%) had the best outcome, whereas the worst survival was seen in those with reduced p27 levels and detectable cytoplasmic p27 (N+C; ≤50%, $P = 0.02$, Log-rank test) (Fig. 6*f*).

53 breast cancers were stained with phospho-Akt antibody (Fig. 6*a–c*, right). Normal quiescent breast acini, lymphocytes and stroma showed no Akt-P staining. None of 23 tumors with exclusively nuclear p27 showed Akt activation, whereas 28/30 tumors with cytoplasmic p27 showed phospho-Akt reactivity. Detection of cytoplasmic p27 in human breast tumors was highly statistically significantly associated with Akt activation (χ^2, $P < 0.001$).

Discussion

This study suggests that constitutive activation of the PI3K-Akt pathway mediates TGF-β resistance. TGF-β- and IL-6-sensitive cells were rendered resistant by ectopic Akt activation. Akt activity was increased in 184A1L5^R and WM239 cells. Moreover, *PTEN* transfection into WM239 cells, and inhibition of PI3K by LY294002 in TGF-β-resistant cells restored p27 binding and inhibition of cyclin E-cdk2 and G1 arrest by TGF-β.

Whereas PI3K signaling can inhibit p27 transcription or accelerate p27 degradation in different cell types, we show that Akt can bind p27 and phosphorylate T157. Akt phosphorylated p27WT but not p27T157A *in vitro*. Moreover, the reactivity of cellular and transfected p27 with phospho-Akt substrate and p27T157-P antibodies was strongly reduced by Akt inhibition following LY294002 treatment. The p27T157A showed minimal reactivity with the antibody to phosphorylated Akt substrate and none with anti-p27T157-P. Cellular p27 phosphorylation at T157 increased in normal HMECs in association with Akt activation as cells moved from G0 into G1. Moreover, T157 phosphorylation of p27 was two-fold higher in PTEN-deficient WM239 than in WM35 cells, indicating that T157 of p27 is a putative Akt site *in vivo*.

T157 lies within the nuclear localization signal of p27 (NLS, aa 153–166)[30,31]. Our data indicate that an Akt-dependent pathway regulates p27 localization. Although the T157A mutation may facilitate p27 import irrespective of PKB, phosphorylation at this site appears to inhibit nuclear import of p27. Cells with activated Akt showed cytoplasmic p27. LY294002 treatment or expression of p27T157A in cells with activated Akt restored nuclear p27 localization. Moreover, Akt^{DD} transfection led to cytoplasmic mislocalization of p27. p27 phosphorylation by Akt impaired nuclear p27 import *in vitro*. Phosphorylation within or near the NLS has been shown to inhibit nuclear import of other proteins[32]. Thus, as for other Akt substrates p21, and some forkhead transcription factors (refs. 28,34), in cancers the constitutive p27 phosphorylation by Akt may cause a relative cytoplasmic sequestration of p27. This would limit the p27 available to bind to and inhibit cyclin E-cdk2, compromising the arrest response to antiproliferative factors such as TGF-β and IL-6. In contrast, in normal HMECs, T157 phosphorylation of p27 ac-

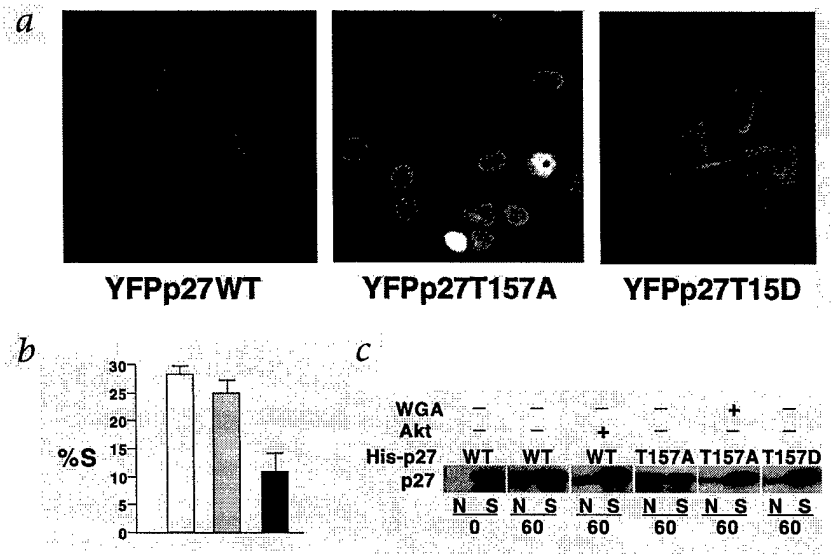

Fig. 5 T157 phosphorylation impairs nuclear import of p27. ***a***, p27 localization following transfection of the indicated p27 alleles into WM35 cells expressing PKB^{DD} (WM35^{PKBDD}). ***b***, WM239 cells were transiently transfected with YFPp27 alleles and the cell-cycle profile of YFP-positive cells analyzed by flow cytometry. □, untransfected; ■, wild-type; ■, T157A-transfected. ***c***, p27 nuclear import was assessed by the addition of His-tagged p27 (His-p27) to digitonin permeabilized cells for 60 minutes (60) and nuclear, (N), and supernatant, (S), fractions were immunoblotted for p27. Input His-p27WT is shown in the left-most lane (t=0 minutes). Pre-treatment of His-p27WT with cellular Akt (+Akt) impaired p27 import. Import reactions for His-p27T157A (T157A) and His-p27T157D (T157D) are shown. Import of T157A was abolished by addition of wheat germ agglutinin (+ WGA).

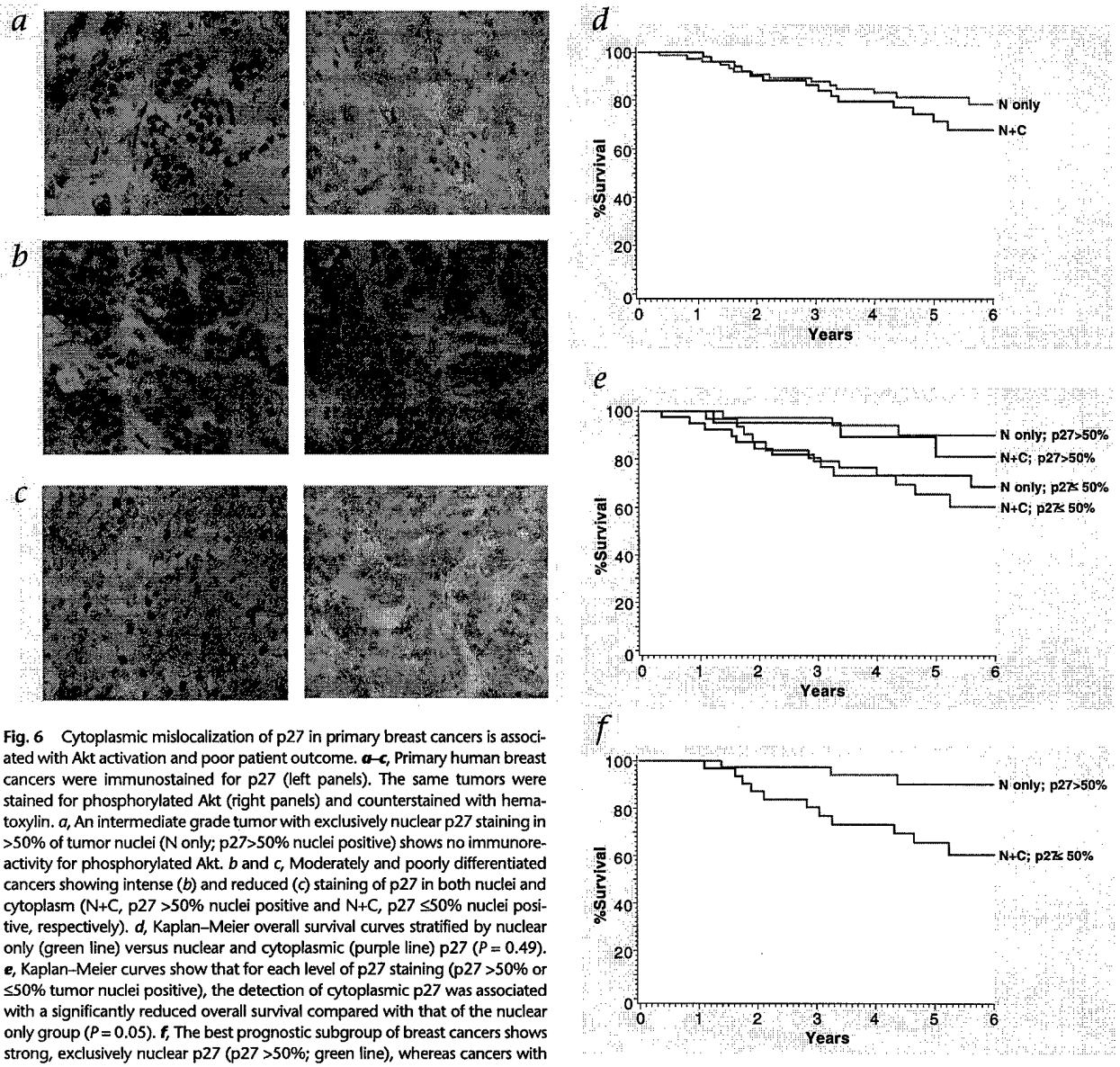

Fig. 6 Cytoplasmic mislocalization of p27 in primary breast cancers is associated with Akt activation and poor patient outcome. ***a–c***, Primary human breast cancers were immunostained for p27 (left panels). The same tumors were stained for phosphorylated Akt (right panels) and counterstained with hematoxylin. *a*, An intermediate grade tumor with exclusively nuclear p27 staining in >50% of tumor nuclei (N only; p27>50% nuclei positive) shows no immunoreactivity for phosphorylated Akt. *b* and *c*, Moderately and poorly differentiated cancers showing intense (*b*) and reduced (*c*) staining of p27 in both nuclei and cytoplasm (N+C, p27 >50% nuclei positive and N+C, p27 ≤50% nuclei positive, respectively). *d*, Kaplan–Meier overall survival curves stratified by nuclear only (green line) versus nuclear and cytoplasmic (purple line) p27 ($P = 0.49$). ***e***, Kaplan–Meier curves show that for each level of p27 staining (p27 >50% or ≤50% tumor nuclei positive), the detection of cytoplasmic p27 was associated with a significantly reduced overall survival compared with that of the nuclear only group ($P = 0.05$). ***f***, The best prognostic subgroup of breast cancers shows strong, exclusively nuclear p27 (p27 >50%; green line), whereas cancers with both reduced levels and cytoplasmic mislocalization of p27 (p27 ≤50%; red line) have the lowest survival ($P = 0.02$).

companies the periodic activation of Akt and may regulate normal p27 function in early G1. It is noteworthy that the PKB consensus sequence in p27 is imperfect and shows some species variation. While PKB may phosphorylate p27 at this site in humans, the possibility that other kinases phosphorylate T157 cannot be excluded.

PKB^{DD} transfection increased cyclin D1 levels in the $WM35^{PKBDD}$ but not in 184^{PKBDD}, but p27 was mislocalized to cytoplasm in both cell types. PKB^{DD} mediated cytoplasmic p27 mislocalization does not result from increased cyclin D1, since cyclin D1 overexpression did not cause cytoplasmic sequestration of p27 in our assays.

Whereas many reports have shown the importance of accelerated p27 proteolysis causing reduced p27 in human cancer[19], only one study showed an effect of cytoplasmic p27 on outcome in esophageal cancer[20]. Patient data in which p27 loss and p27 localization are analyzed together with respect to disease outcome have not been published to date. Here, we demonstrate that Akt-mediated phosphorylation of p27 in its NLS impairs its nuclear import *in vitro* and show that cytoplasmic p27 localization is linked to Akt activation in human breast cancer *in vivo* and is associated with reduced patient survival.

Three independent reports (including this study) demonstrate cytoplasmic p27 in up to 40% of primary human breast cancers in association with activated Akt (Viglietto *et al.* and Shin *et al.* in this issue). Serine 473-phosphorylated Akt was not detected in normal breast epithelium and Akt-P staining showed greater intensity in invasive than in non-invasive tumor areas within individual breast cancers (unpublished data). Tumors with uniquely

nuclear p27 localization showed no Akt activation.

Although Akt can inhibit p27 gene expression by targeting the forkhead transcription factors[25] and loss of *PTEN* may lead to accelerated p27 proteolysis[24], this seems to be cell-type dependent and may require additional changes in signaling pathways. LY294002 increased p27 protein in the melanoma lines but not in normal HMECs. In HMECs and in human breast cancers, Akt activation was not always associated with p27 loss. There was no statistical correlation between cytoplasmic p27 and reduced p27 protein in primary breast cancers. Approximately the same proportion of tumors with high and low p27 scores showed cytoplasmic p27. One possible implication of this is that the processes leading to cytoplasmic p27 mislocalization and accelerated p27 proteolysis may arise independently *in vivo* and each may contribute to tumor progression.

For all levels of p27 staining, the presence of p27 in the tumor-cell cytoplasm was associated with reduced differentiation and lower disease-free survival ($P = 0.003$) and overall survival ($P = 0.05$). These data have implications for the clinical application of p27 as a prognostic factor. Taking into account the presence or absence of cytoplasmic p27 may add to the prognostic significance of reduced p27 levels. Larger studies will be needed to confirm this.

Although one report suggested that p21 phosphorylation by Akt leads to its cytoplasmic sequestration[34], two other groups confirmed that p21 is a Akt substrate, but did not observe cytoplasmic mislocalization of p21 upon Akt activation[35,36]. p21 is expressed more sporadically and at lower levels than p27 in human breast cancers and previous studies have shown conflicting results regarding the prognostic potential of p21 (ref. 37). Although mislocalization of both p21 and p27 could theoretically cooperate to promote tumor progression and a comprehensive study of p21 and p27 levels and localization would be of value, p27 may have greater potential clinical utility in cancer prognosis.

Recent reports suggest that MAPK activation accelerates p27 proteolysis[38,39]. This study, together with reports from Viglietto and Arteaga, suggest that constitutive PI3K-Akt activation contributes to oncogenesis through inhibition of nuclear p27 import and hence its cdk inhibitory function. Cytoplasmic mislocalization of p27 worsens the prognosis associated with reduced p27 levels in breast cancer supporting the relevance of these mechanisms to human tumorigenesis. Although some tumors show evidence of both accelerated p27 proteolysis and cytoplasmic localization, others show only one or the other. In human cancers, mutational activation of *Ras* and loss of the tumor suppressor *PTEN* are not infrequent[21,22]. Overexpression of receptor tyrosine kinases (RTKs), such as Her2, can also activate PI3K-Akt in human breast and other cancers[40]. As both PI3K and MAPK are downstream of RTK-Ras, it will be of interest to determine what additional pathways direct RTK signaling to mediate either p27 proteolysis or cytoplasmic mislocalization in some breast cancers, while in others both coexist.

Akt phosphorylates proteins involved in signal transduction, apoptosis and gene expression[40], and increasing evidence suggests that this pathway contributes importantly to cell-cycle regulation. Although this pathway affects multiple cell-cycle effectors, including cyclin D and p21, the present study, together with those of Arteaga and Viglietto, define a novel mechanism linking Akt activation with impaired nuclear p27 import and p27 deregulation in human cancer.

Methods

Cell culture. Finite life span HMEC strain 184 (ref. 41) and immortalized 184AIL5^R (ref. 12), MCF-7 cells[42] and WM35 and WM239 lines[43] were cultured as described. Cells were treated with 10 ng/ml TGF-β.

Plasmids, site-directed mutagenesis and transfection. The retroviral pBABE vector or pBABE constructs carrying the constitutively activated, hemagglutinin (HA)-tagged PKB$^{T308D/S473D}$ (PKB^{DD})[26,27] or wild-type PTEN were transfected as described[26]. Human wild-type p27 cDNA (p27WT) was inserted into the pIND vector. T157 of p27 was replaced with alanine (p27T157A) or aspartic acid (p27T157D) by site-directed mutagenesis using a QuickChange site-directed mutagenesis kit (Strategene, Loyola, California). The WT and mutant p27 cDNAs were then subcloned into pEYFP-C1 vector (Clontech, Palo Alto, California) encoding an N-terminal yellow-green variant of the *Aequorea victoria* green fluorescent protein (YFP). The YFP-p27 vectors were transfected using LipofectAMINE/PLUS reagents (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. Muristerone A (MA) inducible MEK^{EE} expression was achieved in MCF-7 cells using the 2-plasmid system from Invitrogen. In the absence of MA, cells showed basal MAPK activity. MA increased MAPK-P in 2 different MEK^{EE} inducible lines. pRC-CMV-cyclin D1 (provided by P. Hinds) was transfected into MCF-7 cells to generate cyclin D1–overexpressing cell lines.

Intracellular localization of p27. HMECs were EGF-depleted for 48 h and endogenous p27 detected by immunocytochemistry as described[33]. Nuclear cytoplasmic fractionation was carried out by digitonin-permeabilization as described[44]. Nuclei were isolated by centrifugation and the supernatant containing the cytosolic fraction collected. Equal cell volumes of nuclear and cytoplasmic lysates were assayed for p27 by immunoblotting. The nuclear protein, RCC1, was blotted as a fractionation control. YFP-tagged p27 expression was detected by direct fluorescence microscopy of transfected cells. Photographs were taken at ×400 magnification using an Empix digital camera and 'CoolSnap' (Photomajics, Pittsfield, Massachusetts) software.

Nuclear import assays. Import assays were carried out as described[44]. MCF-7 cells were digitonin permeabilized and isolated nuclei incubated with cytosolic proteins (4 μg/μl), an ATP-regenerating system and recombinant his-tagged WT, T157A or T157D p27 for 60 min at 21 °C. Nuclear and supernatant fractions were then separated by centrifugation and immunoblotted for His-p27. Where indicated, His-p27WT was reacted *in vitro* with cellular Akt before import assays. Pre-incubation with wheat germ agglutinin (200 μg/ml) abolished active p27 import.

Flow cytometry. Cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdU), stained with anti-BrdU-conjugated FITC (Becton Dickinson, Mountain View, California) and propidium iodide and cell-cycle data acquired as described[12].

Recombinant protein, affinity purification and kinase assays. The p27WT, p27T157A and p27T157D cDNAs were subcloned into pET28a. Recombinant His-p27 was purified on nickel-agarose beads. An antibody against serine 473 (Ser473) phosphorylated Akt (ref. 26) was used to assay Akt activation by immunoblotting. For assays of Akt activity, Akt was immunoprecipitated from 1 mg cell lysates and reacted with GSK-3-α as substrate using an Akt kinase kit (New England Biolabs, Beverly, Massachusetts). Akt kinase assays were also carried out using either 5 μg histone-H2B or 10 μg recombinant His-p27 as substrates as described[45]. Cyclin E1-dependent kinase activity was assayed and quantified as described[9].

Antibodies and immunoblotting. Antibodies to Akt, GSK-3-β, phospho-Akt, phospho-GSK3-β (Thr21) were obtained from New England Biolabs; to p27 (C-19) and cdk2 from Santa Cruz Biotechnology (San Cruz, California); to p27 (DCS72) from Neomarkers (Fremont, California); to p27 from Transduction Labs (Lexington, Kentucky); to β-actin from Sigma (Oakville, Ontario). Cyclin E1 antibodies (mAbs E12 and E172) were obtained from E. Harlow. Antiphospho-Akt-substrate antibody was from New England Biolabs. The antibody specific for T157-phosphorylated p27 was generated and provided by G. Viglietto. Cells were lysed in ice-cold NP-40 lysis buffer and immunoblotted as described[12].

Patient population and statistical methods. The population studied was a group of 128 patients who underwent surgery for non-metastatic primary breast cancer between 1986 and 1992 at the Sunnybrook Health Sciences Center. This study was approved by the Research Ethics Board of the hospital. Kaplan-Meier survival and disease free survival curves were generated using nuclear p27 score and nuclear versus nuclear and cytoplasmic p27 as strata. Log-rank, Wilcoxon and –2log (LR) were used to assess significance. The association between discrete variables was tested using the χ^2 test.

Immunohistochemistry. Paraffin sections of tumor blocks were stained for p27 as described[33] using monoclonal p27 antibody (Transduction Labs, Lexington, Kentucky) diluted 1:1000 (0.25 μg/ml) in PBS or for phospho-Akt using the phospho-Akt (Ser473) antibody (NEB) diluted 1:200. Sections were counterstained with hematoxylin. The degree and localization of p27 staining was scored independently by two pathologists (C.C. and J.Z.) and J.M.S. as described[33]. Tumors showing both nuclear and cytoplasmic or exclusively cytoplasmic p27 in at least 35% of cells were scored 'N+C'. Tumors with exclusively nuclear p27 were scored as nuclear only (N only). Phospho-Akt was scored by J.Z. and J.M.S.

Note: Supplementary information is available on the Nature Medicine website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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